FACTORS THAT CHANGE AGGLUTINABILITY OF AGEING SPERM.

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By a previously described technique (Goldforb, '29*a*), agglutination of sperm by egg water could be measured with an average experimental error of 1 second or 4.5 per cent. With this technique freshly shed eggs from freshly collected and freshly tested sea urchins (*Arbacia punctulata*) were separately tested by freshly shed sperm, under strictly comparable conditions. These "normal" germ cells varied from 11 to 2,300 per cent. in agglutination time. This large variation was due in small part to germinal differences and in large part to wide differences in the degree of overripening of the germ cells at the time of shedding.

Later studies (Goldforb, '29*b*) showed that when eggs or sperm or both were not too overripe, at the time of shedding, there was, with ageing, a progressive and marked increase in agglutination time. The evidence compelled the conclusion that ageing eggs liberated increasing amounts of agglutinin, and that ageing sperm either secreted increasing amounts of a substance that increased the agglutination, or, that ageing sperm underwent a physiologic change that made them increasingly susceptible to a given dose of agglutinin.

The present study aims to determine which of these two possibilities actually obtains. The experiments were performed at the Marine Biological Laboratory at Woods Hole, Massachusetts, during the summers of 1924 and 1926. My thanks are due to the Directors for the facilities of the laboratory.

Experiments with Ageing Sperm Suspensions.

In preliminary experiments, samples of the same sperm suspension and the same egg water solution were tested 10 to 50 minutes after the initial test. In a considerable number of instances the later test gave increased agglutination values. Exps. 5, 12, and 25 may serve as illustrations.

TABLE I.

SHOWS AN INCREASE IN AGGLUTINATION TIME WHEN SAMPLES OF THE SAME SPERM AND EGG CULTURES WERE USED 10 AND 20 MINUTES AFTER THE INITIAL TESTS.

Exp. No.	Age of			♀ No.	Agglutination in 1/80 Egg Water.				
	Eggs.	Sperm.	Egg Water.		Initial Test in Sec.	10 Min. Later		20 Min. Later	
						Sec.	% Increase.	Sec.	% Increase.
5A	4 hrs.	4 hrs.	1 hr.	1	11	13	18	19	72
				2	13	14	7	14	7
				3	7	7	0	7	0
				4, 5, 6, 7 [1]	12	16	33	21	75
B	1	22	21	1	24	26+	8		
				2	13	23+	76		
				3	8	26+	225	80	900
				4, 5, 6, 7 [1]	54	57	55	60	111
C	$6\frac{1}{2}$	22	$11\frac{1}{2}$	1	0	0	0		
				2	0	12	1200		
				3	0	13	1300		
				4, 5, 6, 7 [1]	17	17	0		
D_1	22	22	1	1	19	19	0		
				2	9	7	− 22		
				3	9	12	33		
				4, 5, 6, 7 [1]	12	0	0		
D_2	22	22 + 1 [2]	1	1	37	37	0		
				2	0	12	1200		
				3	12	13	7		
				4, 5, 6, 7 [1]	10	19	90		
D_3	22	1	1						
				2	13	12	− 7		
				3	17	11	− 35		
				4, 5, 6, 7 [1]	0	0	0		

[1] Eggs of females 4, 5, 6 and 7 were combined.
[2] Old and fresh sperm combined.

TABLE II.

SHOWS INCREASED AGGLUTINATION VALUES WHEN SAMPLES OF THE SAME OVERRIPE SPERM SUSPENSIONS ARE USED AT SUCCESSIVE INTERVALS.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water Solution.					
	Eggs.	Sperm.	Egg Water.		Initial Test.	10 Min. Later.	15 Min. Later.	35 Min. Later.	55 Min. Later.	% Increase.
12.1	1 hr.	25 hrs.	25 hrs.	1	0				17	1700
				2	0				13	1300
				3	0				18	1800
				4, 5, 6	0				8	800
12.3	1	25	1	1	3			11		269
				2	0			5		500
				3	0			0		0
				4, 5, 6	0			10		1000
12.2	25	25	1	1	0			9		900
				2	0			12		1200
				3	0			0		0
				4, 5, 6	0			16		1600
12.4	1	1	25	1	22			0		− 2200
				2	42			17		− 60
				3	13			9		− 30
				4, 5, 6	17			20		17
12.5	25	1	1	1	22			15		25
				2	18			22+		22
				4, 5, 6	7			24		240

TABLE II.—(*Continued*).

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water Solution.					
	Eggs.	Sperm.	Egg Water.		Initial Test.	10 Min. Later.	15 Min. Later.	35 Min. Later.	55 Min. Later.	% Increase.
12.6	1	1	1	1	0			9		900
				2	6			0		− 600
				3	0			0		0
				4, 5, 6	17			11		− 35
25.3	0	6	1	1	0	0				0
				2	20	30				50
				3	18	23				27
				4	0	0				0
25.6	0	24	1	1	0		0			0
				2	0		28			2800
				3	0		16			1600
25.6	24	24	1	1	0		30			3000
				2	28		34			21
				3	47		40+			
25.6	0	3	1	1	0		11			1100
				2	24		28			16
				3	25		48			92
				4	12		43			258

In Experiment 5*A* (Table I) a fresh suspension of a 4 hour dry sperm was tested separately, with the egg waters of 4 females. The agglutination values were 11, 13, 7, and 12 seconds respectively. Ten minutes later samples of the same cultures gave 2, 1, 0, and 4 seconds *longer agglutinations* than the first tests, or an increase of only 16 per cent. Other samples of the same sperm suspension tested after 10 more minutes gave 8, 1, 0, and 9 seconds more than the initial tests, or an average *increase of* 42 *per cent.*

When a 22 hour sperm was used with freshly shed eggs (Exp. 5*B*) the initial agglutinations for the four females were 24, 13, 8, and 54 seconds respectively. Ten minutes later, samples of the same cultures gave increases of 2, 10, 18, and 3 seconds respectively, or 33 per cent. After 10 more minutes the increases were far greater, namely 126 per cent. When intermediate aged ($6\frac{1}{2}$ hours old) eggs were tested by 22 hours old sperm the first test gave 0, 0, 0, and 17 seconds, the second test 10 minutes later gave 0, 12, 13, and 17 seconds, *i.e.*, an increase of 147 per cent. When, however, freshly shed sperm was used (Exp. *D*3) no increase in agglutination occurred at the later test. When old and fresh sperm were combined (Exp. *D*2) there was again an increase in agglutination values, ten minutes later of 37 per cent.

Similar results occurred in Experiment 12 (Table II). Suspensions of a 25 hour dry sperm did not agglutinate with freshly shed eggs, but 55 minutes later agglutinated 17, 13, 18, and 8 seconds respectively (Exp. 12.1). In Experiment 12.3 the initial values were 3, 0, 0, and 0 seconds. After 35 minutes the values were 11, 5, 0, and 10 seconds respectively, an increase of 766 per cent. When 25 hour old eggs were used, the initial values were 0, 0, 0, and 0 seconds. The later values were 9, 12, 0, and 16 seconds (Exp. 12.2). Ten out of the twelve tests with the 25 hour old sperm gave material increases in agglutination.

On the other hand the freshly prepared suspensions of ripe sperm with ripe eggs, Exps. 12.4 and 12.6, gave little increased or much decreased agglutination. The average values were −13 per cent. and −61 per cent.

It appears that sperm in standard 1 per cent. suspension changed

within 10 minutes, changed further within the next 10 to 45 minutes, with corresponding increase in agglutination values. The change occurred much more markedly in overripe than in ripe sperm. The change occurred whenever the eggs were not so overripe that not enough agglutinin was liberated to activate and agglutinate the sperm.

In Exp. 25 similar results were obtained. The eggs and sperm were in good physiologic condition when shed. The sperm was used when 3, 6, and 24 hours old. Marked increases in agglutination occurred in 10 to 15 minutes after the sperm suspension was prepared. These increases occurred in 6 out of 9 tests. The other 3 tests gave no agglutination in either the first or the second tests. In other instances, when no agglutination occurred in the initial test, the second test gave long agglutinations. The average increases were 39, 39, 113, and 1466 per cent. respectively.

Other experiments corroborated these results and led to a more detailed study of ageing suspensions of sperm.

In Experiment 11*A* (Table III) both kinds of germ cells were six hours old. Six females were used. Females Nos. 1, 2, and 3 were tested separately, 4, 5, and 6 together. The temperature was 21° C. with an increase of $\frac{1}{2}$° C. during the $2\frac{1}{2}$ hours of the experiment. Tests were made 15 to 30 minutes apart, with samples of the same sperm suspension and of the same egg water solution. The successive agglutination tests for female No. 1 were 13, 15, 19, 19, 26, 19, 20, 20, 15, and 16 seconds respectively. *There was an unmistakable increase in values with ageing of the sperm suspension.* Maximum values with egg water of ♀1, occurred not when first tested but 75 minutes later, and the increase was 100 per cent. During the subsequent 75 minutes there was a slow and progressive decrease, which did not reach the initial values at the close of the experiment, 165 minutes later.

The egg waters of females No. 2, No. 3, and Nos. 4, 5, 6 combined gave similar results. The average values for the 4 batches of eggs were 14.5, 17.0, 19.2, 20.2, 19.5, 19.7, 17.0, 15.7, 14.0, and 11.5 seconds at the successive intervals. The increases were 100 per cent. for female 1, 56 per cent. for female 2, 35 per cent.

TABLE III.

INCREASE IN AGGLUTINATION WHEN SAMPLES OF THE SAME EGG WATER WERE TESTED BY SAME SPERM SUSPENSION AT SUCCESSIVE INTERVALS. TEMPERATURE 20° C.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water.										Maximal Increase.
	Eggs.	Sperm.	Egg Water.		0	15	30	50	75	90	105	120	150	165 Min.	
11*A*	6 hr.	6 hr.	1 hr.	1	13	15	19	19	*26*	19	20	20	15	16	100%
				2	16	19	22	21	18	*25*	22	20	19	18	56
				3	14	14	15	15	*18*	6	7	9	7	0	35
				4, 5, 6	15	20	21	26	21	*29*	19	14	15	12	93
				Aver. sec.	14.5	17.0	19.2	20.2	19.5	19.7	17.0	15.7	14.0	11.5	71
				Aver. inc. %		+ 17	+ 31	+ 39	+ 34	+ 36	+ 17	+ 8	− 3	−20	
11*B*	6	31	6	1	0	0	0								
				2	0	0	0								
				3	10	0	0								
				4, 5, 6	16	0	0								

					Agglutination in Sec. in 1/80 Egg Water. Temp. 20½° C.								Maximal Increase.
					0	5	15	25	50	80	100	125 Min.	
8*A*	5	29	1	1	21	50	40	70	*90* +	0			328%
				2	17	0	0	0	0	0			
				3	34	40	50 +	90 +	*95* +	6			179
				Aver. 1 & 3	27.5	35	45	80	92	0			
				Aver. inc. %		+ 27	+ 63	+ 191	+ 236	− 89			
				Sea-water					0	0			
8*B*	5	6	1[1]	1	24		24	29	34	39	37	*53*	120
				2	14		*17*	8 +	11	9	11		
				3	23		22	30	29	29	52	76	230
				Aver. 1 & 3	23.5		23	29.5	31.5	34	44.5	64.5	
				Aver. inc. %			− 2	+ 25	+ 34	+ 44	+ 89	+ 174	

[1] Not same egg water solution as in 8*A*.

TABLE III.—(*Continued*).

RANGE OF TEMPERATURE FROM 20½° C. TO 20° C.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water at																Maximal Increase.
	Eggs.	Sperm.	Egg Water.		0	10	20	30	45	55	70	100	120	140	160	180	200	240	260	280	305 Min.	
14*A*	1 hr.	24 hr.	1 hr.	1	30	30	36	48	53	45	60	70	80	62	42	36	26	3	0	0	0	166%
				2	26	26	28	31	43	40	46	42	40	30	27	24	19	6	0	0	0	77
				Aver.	23	23	32	39.5	48	42.5	53	56	60	46	34.5	30	22.5	4.5	0	0	0	
				Aver. inc. %	0	0	+39	+72	+108	+85	+130	+143	+160	+56	+50	+32	−2	−80	−100	−100	−100	
B[1]	1	1	1	1	29	28	21	19	20		18	17	15	11	12	11	12	10	10	10	10	0
				2	21	25	26	23	23		20	21	20	16	15	16	16	16	15	11	11	24
				Aver.	25	26.5	23.5	21	21.5		19.5	19 5	17.5	13.5	13.5	13.5	14	13	12.5	10.5	10.5	
				Aver. change %	0	+6	−6	−16	−14		−22	−22	−30	−46	−46	−46	−44	−48	−50	−58	−58	

[1] Same egg water as in *A*.

for female 3, and 93 per cent. for females 4, 5, and 6. The average increase was 71 per cent. In every instance the maximal values did not take place at the initial test but 75 to 90 minutes later. When the experiment was terminated, after 165 minutes, the values were greater than the initial test in 2 batches,

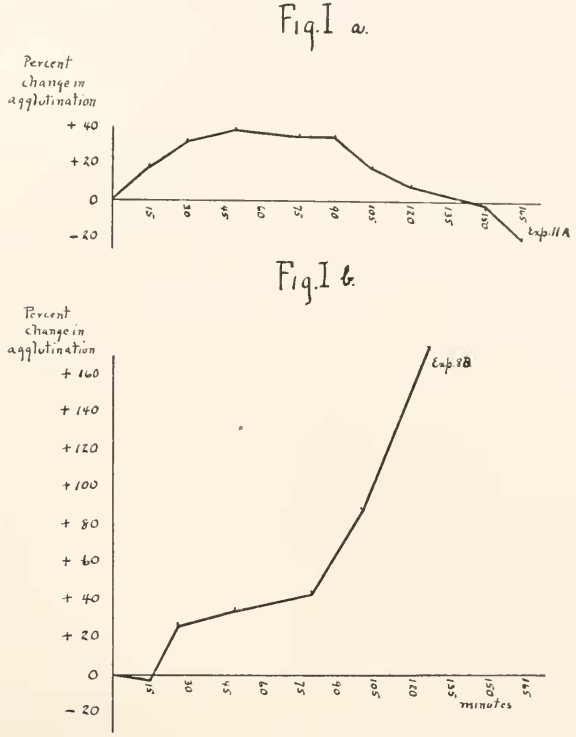

FIG. 1. Shows the slow progressive *decrease* in agglutination values when a suspension of freshly shed sperm is tested at successive intervals with samples of the same egg water.

slightly lower in 1 batch, and in 1 batch agglutination had ceased altogether (Fig. 1*a*).

In Experiment 8*B* the eggs were 5 hours old. These were tested by 2 kinds of dry sperm, one 6 hours old and the other 29 hours old. The temperature increased but $\frac{1}{2}$° C. during the 2 hours of the experiment. When a suspension[1] of the 6 hour dry sperm was tested with the egg water of female No. 1, the

[1] All suspensions of sperm were 1 per cent. and tested immediately.

successive values were 24, 24, 29, 34, 39, 37, and 53+ seconds, respectively. A similar increase occurred with the egg water of female No. 3, namely 23, 22, 30, 29, 29, 52, and 76 seconds. The eggs of female No. 2 were very overripe at the time of shedding, as indicated by enlarged size, oval shape, pale color, greater viscosity, rate of membrane formation, etc. The egg water of these overripe eggs gave a very small increase, then decreased in value, namely 14, 17, 8+, 11, 9, and 11 seconds.

When eggs were not too overripe at the time of shedding, as in female No. 1 and No. 3, there was a progressive and marked increase in values with ageing of sperm suspension, namely 120 per cent. for female No. 1, 230 per cent. in female No. 3. The increase began 10 to 25 minutes after the initial test. The maximum values were not reached during the 125 minutes of the experiment (Fig. 1*b*).

Other samples of the same egg water were tested by a sperm suspension made with a 29 hour dry sperm (Exp. 8*A*). The agglutination values increased to a far greater extent than with the less overripe sperm of the previous experiment. This is in entire accord with the results obtained in ageing dry sperm (Goldforb, '29*b*). The values with the egg water of female No. 1 were 21, 30+, 40+, 70+, 90, and 0 seconds respectively. Female No. 3 gave 34, 40, 50+, 90+, 95+, and 6 seconds respectively. The increases were 328 and 179 per cent. Maximum values occurred in both, 50 minutes after the initial test. There was a very rapid decrease in values after this maximum. Female No. 2, with the very overripe eggs, gave a 17 second agglutination in the first test but no agglutination thereafter (Fig. 2).

It may be concluded from these experiments that *when eggs and sperm were not too overripe, when shed, agglutination progressively increased with ageing of the sperm suspension. The increase was more rapid and reached a greater maximum, the more overripe the dry sperm at the initial test. The change in values cannot be attributed to a change in concentration of sperm suspension, nor to a difference in agglutinin content, nor to ageing of egg water solution.* For, when such egg water was tested at successive intervals by freshly shed, freshly prepared sperm, there was no progressive increase in agglutination values. Maximal

agglutination with ageing sperm occurred not at the initial test but 50 to 125 minutes later. The increase was 35 to 328 per cent.

Ageing sperm suspensions gave a similar, progressive and marked increase in agglutination values as did ageing of dry sperm (Goldforb, '29*b*). Ageing sperm suspensions gave, however, a much quicker increase.

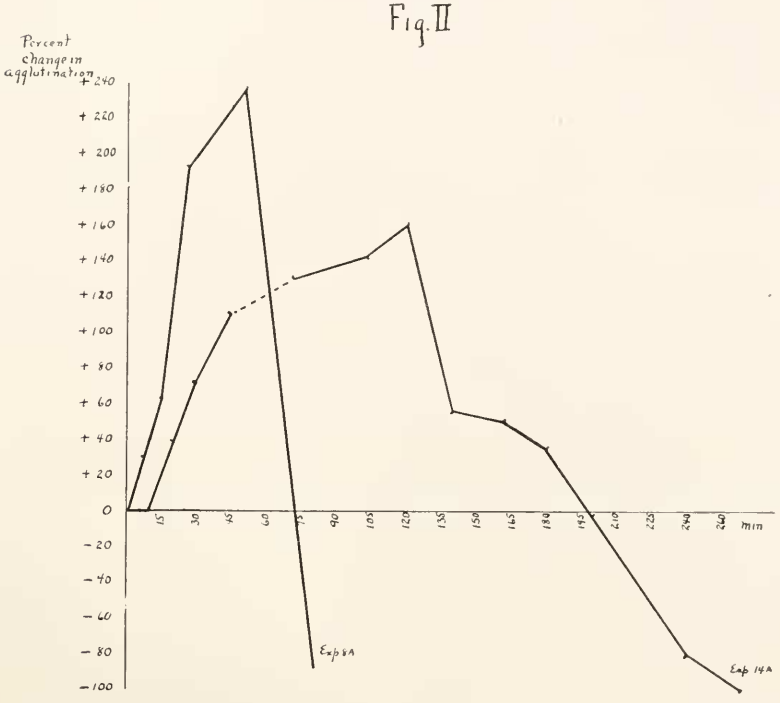

FIG. 2. Shows the slowly *increasing* agglutination values, when partially overripe (6 hours old) dry sperm is used.

In Experiment 14*A*, the ageing sperm suspension was tested for a longer period (305 minutes). Two kinds of sperm were used, freshly shed and 24 hour dry sperm. These were tested by the same egg water solutions from the freshly shed eggs of two females. The egg water was more diluted (1/320) than in the other experiments, which made for greater accuracy. The temperature changed but $\frac{1}{2}^{\circ}$ C. during the five hours of the experiment. Successive tests were made 10 to 25 minutes apart.

The results obtained with the different samples of the same

ageing suspensions of overripe (24 hour) dry sperm conform in all essentials with those in previous experiments. There was a marked and progressive increase in agglutination with ageing of sperm suspension. This increase began 10 to 20 minutes after the initial tests. Maximal values were reached 120 and 70 minutes after the first tests, and were 166 and 77 per cent. greater. Thereafter the values decreased steadily. When the sperm suspension was 260 minutes old, agglutination ceased (Fig. 2).

The parallel experiment with samples of the same egg water solution but tested with non-overripe (freshly shed) sperm gave very different results. Female No. 1 gave at successive intervals

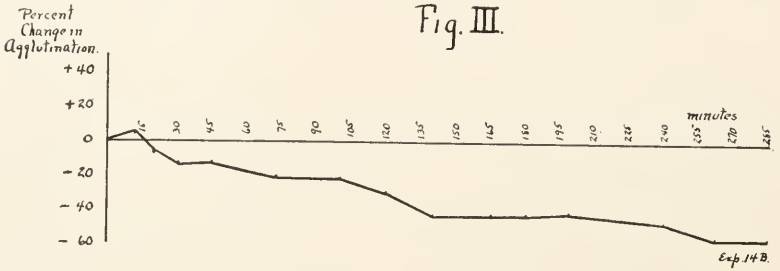

FIG. 3. These curves represent the behavior of dry sperm 24 and 29 hours old. The ageing suspension gave rise to a *more rapid and greater increase* than 6 hour dry sperm. The more rapid and greater increase in 8*A* may be due in part to the older dry sperm.

no progressive increase as in the case of overripe sperm, but on the contrary a progressive decrease, as described by Lillie, '14, '15, Cohn, '18, Lillie and Just, '24. The values were 29, 28, 21, 19, 20, 18, 17, 15, 11, 12, 11, 12, 10, 10, 10, and 10 seconds respectively. The values for female No. 2 showed the same progressive decrease, after a brief, small increase. This small and early increase of 6 per cent. is probably of no significance. The values were 21, 25, 26, 23, 23, 20, 21, 20, 16, 15, 16, 16, 16, 15, 11, and 11 seconds respectively (Fig. 3).

Sperm too overripe, *i.e.*, 31 hour dry sperm (Exp. 11) did not agglutinate at all.

The data are plotted in Figs. 1 to 3.

The increase in agglutination either did not occur or only slightly, when the dry sperm were not overripe at the beginning of the experiment. Nor did it occur when the sperm or the

eggs were so senescent that agglutination did not occur at all, as in Exp. 11*B* and in female No. 2 of Exp. 8*A*. But agglutination did increase when the dry sperm was in intermediate stages of overripeness.

The close agreement in the results with ageing dry sperm (Goldforb, '29*b*) and with ageing sperm suspensions is most striking. The difference lies only in the rate of increase which is so much faster in ageing sperm suspensions than in ageing dry sperm.[1]

It is known that sea water dilutes the H ion concentration of the sperm culture, thereby activating the sperm. But activation by sea water neither gave rise to, nor increased, the agglutination values. Hence the increase in values with ageing of sperm suspension must be attributed to causes other than H ion concentration *per se*.

In searching for the cause or causes of this phenomenon I have excluded an increase in agglutinin as a factor. For the same egg water was used in successive tests, and there were neither eggs nor visible jelly in such solutions. I have excluded the effect ageing of the egg water solution. For when such egg water solution was tested at each successive interval by freshly prepared ripe sperm there was no progressive increase in agglutination values. Temperature was eliminated as a factor, for not only was the change but $\frac{1}{2}$° C., but the increase in agglutination occurred both when the temperature increased and when it decreased. A change in concentration of sea water was also eliminated.

The factor or factors that made for increasing agglutination must be sought in the sperm. If the sperm secretes a substance with ageing, it is not one which activates the eggs to greater agglutination liberation. For there were no eggs in the solution. Nor is there any evidence that it progressively activates the agglutinin in the solution. The other possibility is that sperm undergoes with ageing a physiologic alteration which makes the sperm increasingly susceptible to a given dose of agglutinin. The change is a cyclical one increasing with age, reaching a maximum long after the initial test, and decreasing with further ageing.

[1] Drzewina, A., and Bohn, G. ('26), found that an increasing number of eggs (*Strongylocentrotus*) develop when dilute sperm stood for a time.

The parallelism between the known physiologic changes and ageing of sperm is most striking, such as longevity, viscosity, mobility, metabolism, permeability, agglutination. These physiologic changes are all cyclical. The agglutination increases with overripening of sperm either dry or in suspension. The dry sperm undergoes slower physiologic changes than do sperm in suspension, and shows correspondingly slower increase in agglutination values.

Is There an Increase of Sperm Substance in Increasing Concentrations of Sperm?

A number of investigators have successfully extracted a substance or group of substances from sperm. Winkler, '00, Robertson, '12, Foa, '18, Prevost and Dumas, '24, extracted a sperm substance which induced parthenogenesis. Sampson, '26, described the chemico-physical properties of a sperm filtrate which induced parthenogenesis. Dubois, '00, extracted a spermase, Ostwald, '07, a peroxidase and a catalaze. Geis, '01, and Loeb, '06, were unable to find enzyme characteristics in the sperm extract, but Richards and Woodward, '16, did. Lillie, '15, and Cohn, '18, suggested the possibility that overripe sperm may liberate a substance which aids in fertilization. Popa, '27, described a lipochromatic substance in the sperm head, which substance he believes responsible for agglutination.

Such investigations strongly suggested that overripe sperm may secrete a substance which modifies agglutination. While the evidence from ageing sperm strongly pointed to a physiologic change in the sperm, the hypothesis of increasing liberation of sperm substances with overripening was not excluded.

To find out whether the sperm liberated a substance which progressively increased agglutination, experiments were made with increasing concentrations of sperm, but with samples of the same egg water. The concentrations of sperm ranged from $\frac{1}{2}$ per cent. to 25 per cent. More than 25 per cent. could not be used for the suspensions were then too opaque to distinguish agglutinated clusters in the thick creamy mass of sperm. If the increase in agglutination in previous experiments was due to an increasing liberation of a sperm substance which energized

agglutinin, one should expect that increasing concentrations of sperm should correspondingly lengthen agglutination time. If the increase is due to a physiologic change in the sperm, then an increase in concentration, *per se*, should not increase agglutination.

In Experiment 4*A* (Table IV) ripe sperm was used in $\frac{1}{2}$, 1, 2, and 4 per cent. suspensions. The agglutination values in a 1/20 dilution of egg water were 19, 27, 25, 28 seconds respectively. In a 1/60 egg water dilution, and therefore more accurate, with sperm in $\frac{1}{2}$, 1, 2, 4, 10, and 25 per cent. concentrations, the agglutination values were 7, 14, 13, 13, 13, and 13 seconds respectively. In a still more dilute egg water dilution, namely 1/120, and with sperm in 1, 2, 4, and 10 per cent. concentrations, the values were 9, 9, 10, and 9 seconds respectively. The egg water of female No. 2 in a dilution of 1/120 gave 17, 27, 22+, 27, 28, and 28 seconds.

Less than 1 per cent. concentration of sperm did not give full agglutination values as Lillie has already made clear. At least 1 per cent. suspension is needed. But increasing the concentration of sperm *did not* further increase the agglutination values even in the dense 25 per cent. concentration.

There was the possibility that not enough time had been allowed for the liberation of the hypothetical substance or substances from the sperm. Previous experiments, however, showed that ageing 1 per cent. sperm suspension began to increase in agglutinability within 5 to 25 minutes, and reached maximal values in 15 to 125 minutes. Ageing dry sperm showed the initial increase within 3 hours and gave maximal values in 63 per cent. of the tests when 3 to 4 hours old.

In Experiment 4*B*, therefore, the same dry sperm was used as in the previous experiment, but was now $3\frac{1}{2}$ hours old. Furthermore the sperm suspension was 65 minutes old when used. This was then deemed ample time to liberate the hypothetic sperm substance. With increasing concentration of sperm there should be increasing quantities of the sperm substance, manifested in increasing agglutination values. In a 1/20 dilution of egg water and with 1, 2, 4 per cent. sperm concentrations the values were 28, 27, and 29 seconds respectively. In the more delicate tests

TABLE IV.

SHOWING NO INCREASE IN AGGLUTINATION WITH INCREASING CONCENTRATION OF SPERM. AGGLUTINATION IN SECONDS.

Exp. No.	Age of			♀ No.	Concentration of Sperm in %.						Egg Water Dilution.
	Eggs.	Sperm.	Egg Water.		½	1	2	4	10	25	
4A	1½ hr.	1½ hr.	1 hr.	1	19	27	25	28			1/20
				1	7	14	13	13	13	13	1/60
				1		9	9	10	9		1/120
				2	17	27	22+	27	28	28	1/120
4B	3½	3½	1	1		28	27	29			1/20 Suspension 1 hr. old.
				1		10	9	11	11		1/120 " " " "
19A	1	1 [1]	1	1a	28	26	27	26	27	28	1/320 Aliquot parts
				1b	27	29	29	28	27	28	1/320 of eggs.
19B	1	1 [1]	26	1	17	19	18	18	19	18	1/320
				2	11	20	19	20	21	21	1/320
19C	26	1 [1]	1	1	17	18	18	17	18	20	1/320
				2	19	20	15+	16+	16+	18	1/320

[1] Sperm overripe when shed.

with a 1/120 egg water dilution, and with sperm concentrations of 1, 2, 4, and 10 per cent., the values were 10, 9, 11, and 11 seconds. There was clearly no evidence of a sperm substance, even after the lapse of so much time, in any of the concentrations of sperm used.

Experiment 19 is an example of another type of experiment. The sperm, when shed, were by various tests shown to be overripe (Goldforb, '29*a*, '29*b*). This overripe sperm was then tested by (*a*) egg water from freshly shed ripe eggs, (*b*) other freshly shed ripe eggs whose egg water was 26 hours old, (*c*) fresh egg water from overripe (26 hour old) eggs. If a substance is liberated by overripe sperm, it should manifest its presence in one or more of these three tests with overripe sperm. The egg water dilution was 1/320 throughout. The temperature was $22\frac{1}{2}^{\circ}$ C. The sperm concentrations were $\frac{1}{2}$, 1, 2, 4, 10, and 25 per cent., freshly prepared for each test.

In Experiment 19*A* the freshly shed ripe eggs were divided into two equal portions. The egg water of each was tested separately with freshly prepared sperm suspensions. This served to check the accuracy of the experimental method. One portion gave, in increasing concentrations of sperm, 28, 26, 27, 26, 27, and 28 seconds, the other registered 27, 29, 29, 28, 27, and 28 seconds. There is a remarkably close agreement in the two samples of eggs. There was clearly no evidence of a sperm substance which increased the duration of the agglutination phenomenon.

Comparison of Experiments 19*A*, 4*A*, and 4*B* with those experiments in which ageing sperm were used, brings out in sharpest relief the complete absence of any increase in agglutination in either ripe or in overripe sperm by merely increasing the concentration of the sperm, while progressive and marked increases occurred in 1 per cent. suspensions as they became increasingly overripe.

In Experiment 19*B* old egg water (26 hours old) was used, with the same overripe sperm, in the same concentrations. The egg water of female No. 1 gave 17, 19, 18, 18, 19, and 18 seconds respectively. Female No. 2 gave an 11 second reaction in ½ per cent. sperm suspension, and 20, 19, 20, 21 and 21, seconds in

the other concentrations. The heavy concentrations of sperm cultures did not manifest any increase in agglutination in these old egg water solutions.

In Experiment 19*C* overripe (26 hour) eggs were used. Female No. 1 gave, in the increasing sperm concentrations, 17, 18, 18, 17, 18, and 20 seconds. Female No. 2 gave 19, 20, 15+, 16+, 16+, and 18 seconds. There was no increase in values when overripe eggs were used.

In Experiment 19 with ripe or overripe eggs, with fresh and with old egg water solutions, with ripe or overripe sperm, *there is no evidence of a substance liberated by sperm.*

Discussion.

There can be no doubt that agglutination was not increased with increasing concentration of sperm. On the other hand agglutination was markedly and progressively increased by ageing of sperm, either concentrated or in suspension. When increased agglutination took place, it was *not* due to a substance activating the eggs to greater agglutinin production. For the increase in agglutination values occurred in samples of the same egg water from which eggs and jelly were excluded.

It is conceivable that the substance may be modified so as to intensify the activity of the agglutinin in the egg water. This was suggested as a possibility by F. Lillie, '19. In the first place there is no known basis for this hypothesis. Much more pertinent is the fact that one should expect on this hypothesis an increase in agglutination values in those experiments in which increasing concentrations of sperm were used. But no such increase occurred.

It should be recalled that students of the agglutination phenomenon in bacteria have come to a similar conclusion, namely, that a physiologic change or changes in the bacteria are responsible for the change in agglutination (McGregor, '10, Ficai, '12, Kabeshima, '13, Buchanan, '19).

The observation that loss of fertilization occurs more quickly than loss of motility (Lillie, F., '14, '15, '19, Lillie, F., and Just, E., '24) is paralleled by the observed fact that loss of agglutination occurs more quickly than loss of motility. It is conceivable

and probable that loss of fertilization and of agglutination are associated with the physiologic changes described above.

Sufficient time elapsed to permit the substance, if present, to be liberated into the culture medium. Ripe eggs liberate a substance, agglutinin, in about 15 minutes. Sperm on account of their small size and relatively large surface should liberate their substance more quickly. Yet in 3½ hours, ripe dry sperm did not give any evidence of a substance that increases agglutination, nor did the overripe sperm in Experiments 19*A*, 19*B*, 19*C*.

Nor may one assume that the hypothetic activating substance is formed and liberated increasingly with ageing sperm. For even in overripe sperm, Experiment 4*B*, the increasing concentrations give no evidence of increase in agglutination.

It is known that sperm give off CO_2, and the carbonic acid thus formed modifies the relative OH ion concentration of the medium. The work of Loeb, '03, '14, Lillie, '14, '15, Cohn, 18, Lillie and Just, '24, clearly indicates that the carbonic acid' plays an important rôle in *decreasing the activity and thereby increasing the longevity of the sperm*. It is known that *carbonic acid induces aggregation but there is no evidence of carbonic acid increasing agglutination*.

It is therefore concluded that carbonic acid is not responsible for the increasing agglutination values.

It is furthermore concluded that there is no definite evidence of a substance liberated by sperm which increases the intensity or the duration of agglutination.

The facts strongly suggest a cyclical physiologic change or changes in the sperm, which make the sperm more reactive to a given dose of agglutinin.

It is known that both eggs and sperm undergo cyclical changes. This is evidenced by changes in metabolism, in gelation of surface, in viscosity, in permeability, etc., all of which are associated with overripening. Overripening eggs show progressive solution of jelly, liberation of agglutinin, or increased rate of agglutinin production. Hence overripening eggs give rise to increased agglutination values. Overripening sperm, undergoing similar physiologic cyclical changes, increases in agglutinability.

When therefore ageing eggs are tested by ageing sperm the agglutination values are greater than when either eggs or sperm alone are aged. This is true not only for ageing dry sperm but for ageing sperm suspensions.

It is this physiologic change in sperm as well as in eggs that gives rise to the initial improving stage, evidenced here in increasing agglutination. This physiologic change, continuing, leads to an optimum condition of the germ cells and then to senescence.

The cyclical physiologic change in eggs is manifested by the cyclical increase then decrease in agglutinin liberation. The cyclical physiologic change in sperm is manifested by increase then decrease in agglutinability.

The life cycle of the sperm may be abbreviated, *i.e.*, the physiologic changes may be hastened by dilution, by heat, by excess OH ions, etc. With such increase there is a corresponding precocious increase in agglutinability. Freshly matured dry sperm may not show evidences of a change for 3 hours. Overripe dry sperm show evidences of a physiologic change at once. Suspensions of ripe sperm show little evidence of a physiologic change during the whole life of the sperm.[1] Moderately overripe sperm in the same dilution show evidences of a physiologic change in 15 to 25 minutes (Exp. 8*B*, 11*A*). Sperm more overripe show evidence of a physiologic change sooner, namely, 5 to 20 minutes (Exp. 8*A*, 14*A*).

If the factor which gives rise to increased agglutinability be a sperm secretion, there should occur with increasing concentration of sperm correspondingly increased agglutination. This does not take place. On the other hand, if the factor be a physiologic change in the sperm, then increasing concentration of freshly prepared sperm should produce no change in agglutination, which is exactly what takes place.

I am therefore compelled to conclude that the increase in agglutination so marked in ageing sperm, whether dry or in suspension, is due to a physiologic change in the sperm, which change makes them more susceptible to a given dose of agglutinin.

[1] It is improbable that the change may occur within the first 10 minutes.

The discovery of agglutinins secreted by the eggs led to the assumption that the egg plays the dominant rôle in agglutination.[1] Lillie, '17, dealing with other phases of germ cell behavior, states that "the old idea that sperm supplies organs or substances necessary for activation must be abandoned. The egg possesses all substances needed for activation. The sperm is an inciting cause of these reactions within the egg system. . . ." It has been assumed that secretions of varying amounts of agglutinin were the determining factor in changing agglutination. The sperm merely reacted to varying quantities of agglutinin.

My studies have shown that the egg does not decrease, but on the contrary *increases the rate of agglutinin liberation* with age until an optimum is reached several hours after maturation. These studies also showed that *sperm are not constant* as heretofore assumed, but that sperm is equally variable, increasing in agglutinability with overripening, until an optimum is reached 6 to 24 hours after maturation (dry sperm) or 70 to 120 minutes after preparation of the suspension. This cyclical change in agglutinability appears not to be due to a secretion, but to a physiologic change which makes sperm increasingly agglutinable by a given dose of agglutinin.

Summary.

Previous studies demonstrated that with increasing overripening of eggs or of sperm, or both, agglutination values increased correspondingly.

The present study demonstrates that precocious overripening of sperm, by dilution, gave rise to a correspondingly precocious and markedly progressive increase in agglutination.

This precocious increase occurred when the dry sperm were overripe. The increase began in 5 to 20 minutes after the initial test. Maximum values occurred 15 to 125 minutes after the initial test. The increase in values ranged from 77 to 328 per cent. The greater the overripeness of the dry sperm the greater the increase, the earlier the maximum and the sooner the cycle ended.

[1] For full bibliography and review I refer to Lillie, Problems of Fertilization, '19, Lillie and Just in General Cytology, '24, and to Morgan, Experimental Embryology, '27.

Suspensions made of ripe sperm either did not increase at all or only slightly. The agglutination values then decreased progressively. This is the phase heretofore described.

The cyclical increase and decrease in agglutination was not due to a change in the eggs, nor jelly, nor temperature, nor to a changed OH ion concentration.

This increase in agglutination is not due to a substance liberated by sperm. For agglutination values were not increased when the concentration of sperm was increased from 1 to 25 per cent., the maximum concentration usable. The values were the same whether ripe or overripe sperm were used.

Sufficient time elapsed for the substance, if present, to be liberated.

The CO_2 liberated by sperm has considerable effect upon the activity of the sperm, upon aggregation, but does not increase agglutination.

The cyclical agglutination change is due to a physiologic change in ageing sperm, manifested in a changing metabolism, gelation, viscosity, permeability, and in an increased reaction of sperm to a given dose of agglutinin.

This cyclical change is paralleled by the eggs, which increases agglutinin liberation.

It is therefore concluded that the cyclical physiologic change in overripening is responsible for the improving phase in both germ cells, and with subsequent senescence. This physiologic change is analogous to that in agglutinating bacteria.

Sperm are not a biologic constant, as heretofore believed, but undergo marked physiologic changes with corresponding marked and progressive increase in agglutinability followed by progressively decreasing agglutinability.

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